

Docket No.: 1422-0679PUS1  
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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IN RE APPLICATION OF:

Yoshio TSUJINO et al.

Application No.: 10/540,156

Confirmation No.: 6601

Filed: June 21, 2005

Art Unit: 1651

For: NEUTRAL PHENOL OXIDASE

EXAMINER: KOSAR,  
Aaron J.

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DECLARATION UNDER 37 C.F.R. 1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Kaori SAITO, residing at Osaka-fu, Japan, hereby declare and state as follows:

1. I am thoroughly familiar with the contents of U.S. Application Serial No. 10/540,156 filed on June 21, 2005, its prosecution before the United States Patent and Trademark Office and the references cited therein.
2. I am a graduate of Japan Advanced Institute of Science and Technology, School of Material Science in the year 2007, majoring in enzymology.
3. I have been employed in MANDOM CORPORATION in the year 2000 and have been assigned to the Research Laboratories.
4. I have been involved in the research and development of characterization of a new multicopper oxidase and its application to hair dyeing since the year 2003.
5. That the following experiments were conducted by myself or under my direct supervision and control in order to verify that the laccase from

*Flammulina velutipes* IFO 30601 of the present invention is distinguishable from the laccase from *Flammulina velutipes* reported in Lee and Suh (*Kor. J. Mycol.* 13(2), 111-114, 1985).

Concretely, the laccase from *Flammulina velutipes* IFO 30601 of the present invention (hereinafter simply referred to as "laccase of the present invention") is distinguishable from the laccase from *Flammulina velutipes* of Lee et al. (hereinafter simply referred to as "laccase of Lee et al.") in the following features.

1) Culture Conditions

Lee et al. discloses that the laccase is obtained by culturing *Flammulina velutipes* in an onion medium, a sawdust medium, and a barley straw medium (pH not listed). On the other hand, according to the present invention, the enzyme activity is improved by using enzyme-inducing medium of alkaline pH.

2) Characteristics of Culture Media

The laccase in the culture medium of Lee et al. has a  $K_m$  value of 28 mM, and the laccase of the present invention, to the contrary, has a  $K_m$  value, as determined hereinbelow, of 0.33 mM.

The procedures taken for obtaining a  $K_m$  value of the laccase of the present invention are described hereinbelow.

## EXPERIMENTAL METHOD

Citric acid and phosphoric acid were dissolved in ion-exchanged distilled water, and the pH of the solution was adjusted to 6.45 with an aqueous sodium

hydroxide, to prepare a 0.2 M buffer. *p*-Phenylenediamine was dissolved in the citrate phosphate buffer (0.2 M, pH 6.45), so as to have a concentration of 10 mM, to give a substrate solution. The buffer, the substrate solution, and the enzyme solution were mixed in a cuvette so as to have final concentrations of the buffer of 0.198 M and *p*-phenylenediamine of 0.1 to 4.0 mM, and a change in absorbance at a wavelength of 525 nm of the mixed solution was determined. An amount of enzyme that elevates one unit of the absorbance in one minute was obtained as an activity of 1 U, and thereafter a Lineweaver-Burk plotting was carried out to calculate a  $K_m$  value.

## RESULTS

A Lineweaver-Burk plot of an activity  $v$  of the laccase of the present invention against a substrate concentration  $s$  is shown in Figure 1. An X-intercept with the straight line is  $1/K_m^{-1}$ , and the  $K_m$  value of the laccase of the present invention is found to be 0.33 mM according to the calculation.

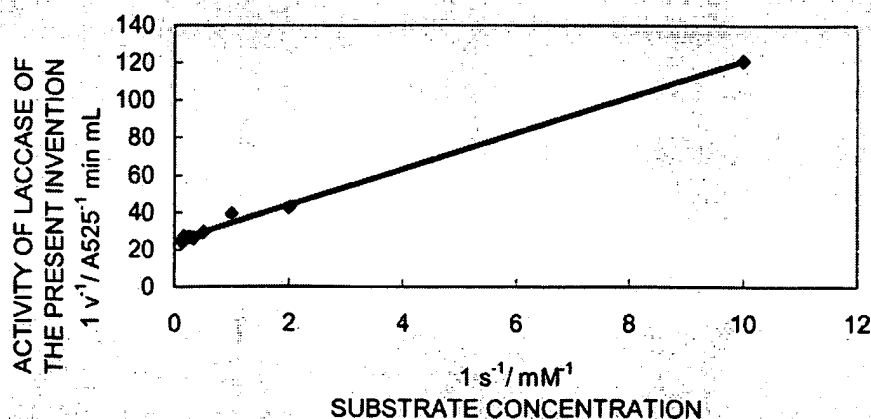


Figure 1: Lineweaver-Burk Plot of Activity of Laccase from *F. velutipes* IFO 30601 Against Substrate Concentration

In conclusion, while the literature value of the  $K_m$  value of the laccase of Lee is 28 mM, the found  $K_m$  value of the specified laccase of the present invention is found to be 0.33 mM, thereby showing that the laccase of the present invention is clearly distinguishable from the laccase of Lee.

6. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

7. Further declarant saith not.

Kaori Saito

Kaori SAITO

December 25, 2007

Date